Role of the Hedgehog/Patched Signaling Pathway in Oncogenesis

A New Polymorphism in the PTCH Gene in Ovarian Fibroma

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ABSTRACT: **We compared the expression of target genes of Hedgehog/Patched signaling in ovarian fibromas and ovarian dermoids. We noted that high levels of SHH appear almost regularly, especially in dermoids, usually accompanied by increased expression of SMO. GLI overexpression does not coincide with that of PTCH. Loss of heterozygosity findings in the PTCH locus and increased expression of several genes in the pathway strongly suggest that the pathway is involved in both ovarian fibroma and dermoids.**

KEYWORDS: **Hedgehog/Patched signaling; SHH; SMO; GLI1; PTCH; PTCH polymorphisms; ovarian fibromas; ovarian dermoids**

INTRODUCTION

The Hedgehog/Patched signaling pathway plays a prominent role during mammalian development, especially during embryonic development, but it is also involved in pathological conditions, including oncogenic transformation. The pathway is strongly conserved through evolution.

Gorlin syndrome is a heritable disease associated with mutations in the human homolog of Patched (PTCH1), which predisposes to multiple skin, brain, and ovarian tumors and to a variety of other malformations.[1] Loss of heterozygosity for the PTCH region was found not only in syndrome-associated tumors but also in nonmalignant malformations (e.g., in the epithelial lining of odontogenic cysts),[2,3] confirming the expectation that the gene responsible for Gorlin syndrome would have an important role in development and organogenesis.[4] PTCH is the only known tumor suppressor mutated both in tumors and in malformations in humans and the only one with the protein located on the cell membrane.

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Activation of the Hedgehog/Patched pathway is initiated through binding of the secreted Hedgehog ligand Hh (i.e., any of its mammalian homologs: Shh, Dhh, or Ihh) to its membrane receptor Ptch (12-transmembrane domain protein). In the absence of Hh, Ptch and Smo (another membrane protein that bears some structural similarities to G-protein-coupled receptors) form an inactive complex. But when Hh binds to Ptch, this relieves the coreceptor Smo, which was repressed by Ptch, and activates a cascade that leads to translocation of the active form of the transcription factor Gli to the nucleus.[5,6] Because it also belongs to the pathway target genes, PTCH acts as a feedback regulator of Hedgehog/Patched signaling, having dual roles in sequestering and transducing Hh.

When the second large extracellular loop, which is essential for ligand binding, is deleted by a PTCH mutation, Hh binding to Ptch cannot occur, but repression of Smo is unaffected. When a C-terminal truncation is caused by a PTCH mutation, Ptch can no longer repress Smo, but Hh binding to Ptch is unaffected[7] (FIG. 1).

The zinc finger transcription factor Gli1 mediates Shh signaling during development, but its expression is also found in several human tumors, including basal cell carcinomas, medulloblastomas, and sarcomas. Many Gli1 targets are associated with

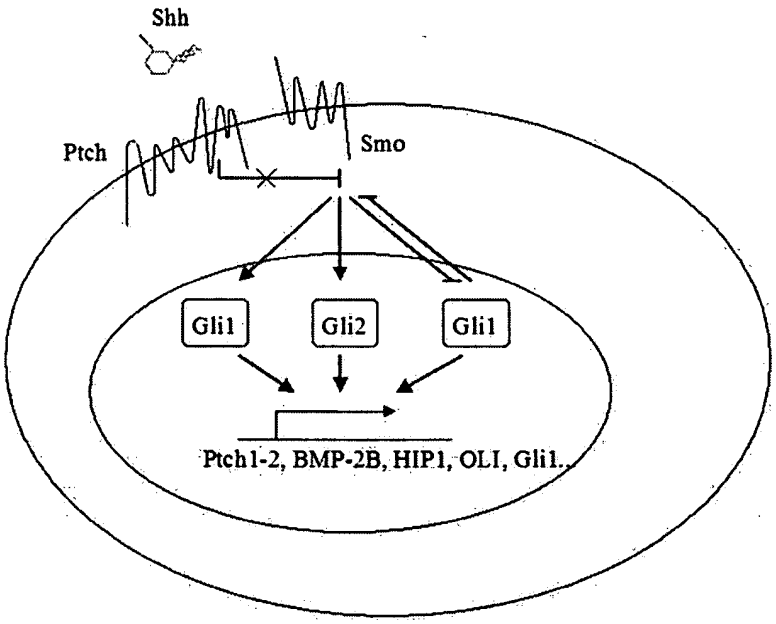

FIGURE 1. Signaling through the Hedgehog/Patched pathway. Secreted ligand Sonic hedgehog (Shh) binds to transmembrane protein Patched (Ptch), thus relieving Smoothened (Smo), another transmembrane protein. Smo transmits the signal to the cytosol, to downstream components of the pathway signaling, activating a cascade that leads to the translocation of the active form of the transcription factor Gli to the nucleus. Nuclear Gli activates target gene expression, including PTCH and GLI itself. Other target genes that are important for the oncogenic function of the pathway are those involved in controlling cell proliferation and in angiogenesis.

cell proliferation, indicating that this oncogene induces cell transformation through multiple downstream pathways.[8]

It has been hypothesized that malfunctioning of the Hedgehog/Patched pathway may be caused by genetic or epigenetic alterations in any of its members, and mutations in some of them have been found in certain pathological conditions. We propose to compare the expression of the pathway target genes in ovarian fibromas and ovarian dermoids to identify tissue-specific and common patterns characteristic of tumorigenesis by deregulated Hedgehog signaling.

MATERIALS AND METHODS

Tumor Specimens

Tumor tissue had been obtained from the Department of Obstetrics and Gynecology, School of Medicine, University of Zagreb (Zagreb, Croatia). The samples were collected with due consideration for all necessary ethical and legal requirements. The tissue was immersed in RNA (Ambion) solution immediately after surgery and was evaluated histologically before extraction of DNA and RNA by a trained pathologist.

Loss of Heterozygosity and Single-Strand Conformational Polymorphism Analyses

Loss of heterozygosity (LOH) and single-strand conformational polymorphism (SSCP) analyses were performed. Genomic DNA from tumor tissue and blood leukocyte pellets of female patients was amplified by PCR. We used highly polymorphic microsatellite markers, D9S196, D9S287, D9S180, and D9S127, which are located on human chromosome 9q22.3 in the vicinity of the PTCH gene, and intragenic PTCH intra marker. The primer sequences were obtained from the Genome Database (http://www-genome.wi.mit.edu). For LOH analysis, PCR products were analyzed on native 8–12% polyacrylamide gels (1 mm × 32 cm × 40 cm) at 500 V.[2,3]

For SSCP analysis, PTCH exons were amplified by PCR and analyzed using the SSCP approach: PCR products were denatured for 10 min at 42°C in 1× denaturing buffer and loaded on 6–9% native polyacrylamide gels (1 mm × 16 cm × 18 cm) at 250 V.[9] DNA was visualized by silver staining.[10]

Quantitative PCR of SHH, SMO, GLI1, and PTCH

RNA was extracted from ovarian fibroma and dermoid tissues, and 1 μg was reverse transcribed into cDNA. The PCR products were amplified with primer pairs described previously: SHH (forward, 5′-GAAAGCAGAGAACTCGGTGG; reverse, 5′-GGAAAGTGAGGAAGTCGCTG), 170 bp; SMO (forward, 5′-CTGGTACGAG GACGTGGAGG; reverse, 5′-AGGGTGAAGAGCGTGCAGAG), 140 bp; PTCH1 (forward, 5′-TCCTCGTGTGCGCTGTCTTCCTTC; reverse, 5′-CGTCAGAAAGG CCAAAGCAACGTGA), 200 bp; and GLI1 (forward, 5′-GCCGTGTAAAGCTCCA GTGAACACA; reverse, 5′-TCCCACTTTGAGAGGCCCATAGCAAG), 200 bp.[8,11,12]

The level of expression was determined densitometrically (Image Master, VDS) and normalized to ribosomal protein PO as a housekeeping gene and to normal ovarian tissue.[6] Four exons were checked by dHPLC (Laboratorie de Genetique Oncologique,

Institut Bergonie, Bordeaux, France) for 10 samples (a detailed protocol of the PTCH1 dHPLC mutation screening method will be published in detail by P. Gorry). Sequencing was performed by MGM Biotech (Ebersberg, Germany).

RESULTS

LOH and SSCP Analyses

One-third of ovarian fibromas and dermoids exhibit LOH in the vicinity of the PTCH1 locus (TABLE 1). More precisely, in 2 of 11 ovarian fibroma tissues and in 4 of 10 ovarian dermoids, LOH was found (FIG. 2). In all of those samples, SSCP analysis showed a variable pattern compared with constitutional DNA in at least one

TABLE 1. Results of LOH analysis for the 9q22.3 region

	9q22.3				
Sample	D9S127	D9S180	D9S287	PTCH intra	D9S196
1F	het	het	ho	ho	het
2F	het	ho	ho	het	het
3F	?	het	het	ho	ho
4F	ho	LOH	LOH	LOH	LOH
5F	LOH	LOH	LOH	LOH	LOH
6F	ho	het	het	het	het
7F	het	het	het	ho	het
8F	het	het	het	ho	het
9F	het	het	ho	ho	ho
10F	het	ho	ho?	ho	het
11F	ho	het	ho	ho	het
1D	het	het	het	het	het
2D	ho	het	het	het	ho
5D	het	LOH?	het	het	het
7D	ho	het	het	ho	het
10D	ho	het	het	het	ho
11D	ho	het	ho	ho	het/ho
12D	het	LOH	ho	LOH	ho
13D	het/ho	LOH	ho	ho	LOH
14D	LOH	ho	het	het	het
15D	het	het	ho	ho	ho

ABBREVIATIONS: D, dermoid; F, fibroma; ho, homozygous; het, heterozygous.

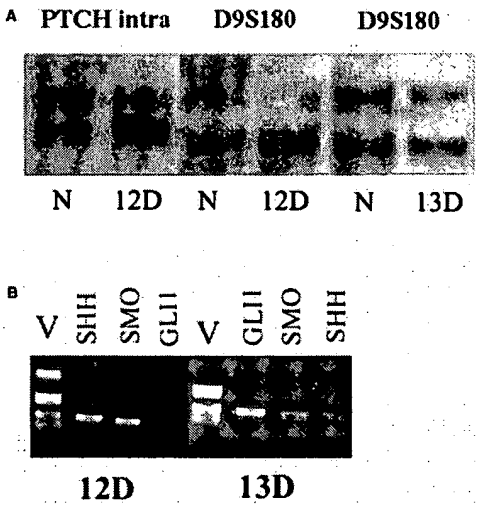

FIGURE 2. (**A**) LOH analysis for polymorphic markers PTCH intra and D9S180 in blood leukocytes (N) and ovarian dermoids (12D and 13D). (**B**) Quantitative RT-PCR analysis of SHH, SMO, and GLI mRNA expression in two ovarian dermoids (12D and 13D). V = DNA molecular weight marker V, 8-587 bp (Roche).

PTCH exon (TABLE 2). A variable SSCP pattern generally indicates mutations or polymorphisms, which can be disclosed by sequencing. Some of these variabilities were sequenced (exons 18 and 21). The results have not yet shown distinct mutations in PTCH, but some polymorphisms were found.

Polymorphisms

Polymorphisms in the PTCH gene were found on exon 1 (2F, 4F, 5F, 1D, 2D, 6D, 12D), exon 11 (D1, D6, D11, D12, D13), exon 12 (1F, 2F, 3F, 5F, 6F, 7F, 9F, 1D, 3D, 5D, 6D, 10D, 14D), exon 15 (2F, 4F, 1D, 2D, 6D, 12D), and exon 23 (D1, D2, D6, D12).

Polymorphisms of Exon 12

Generally, the most frequent polymorphisms of the PTCH gene are observed on exon 12, in particular 1686C→T.[13] This form was also most often detected in our samples: in 1F in both alleles, then in 2F, 6F, 7F and 1D, 6D, 10D, 14 D. The second polymorphism by frequency in the general population[14–16] is 1665T→C; it was found in three of our samples: 5F, 9F, and 3D. The third most frequent polymorphism,[15] 1641C→T, was found in our sample 5D. We also found a new polymorphism that has not yet been published. It is 1647C→T, and it was detected in our sample 3F (FIG. 3).

Expression Levels

The levels of expression of the analyzed pathway genes in tested samples of ovarian fibromas and dermoids are shown in TABLE 3. Variable expression patterns for

TABLE 2. Results of SSCP analysis of PTCH exons 3, 5, 6, 8, 9, 10, 12, 13, 14, 16, 17, 18, 21, and 23

	PTCH													
Sample	Exon 3	Exon 5	Exon 6	Exon 8	Exon 9	Exon 10	Exon 12	Exon 13	Exon 14	Exon 16	Exon 17	Exon 18	Exon 21	Exon 23
1F	N	N	N	N	N	N	N	N	N	N	N	–	–	–
2F	V	N	V	V	V	V	V	V	V	V	N	V	V	N
3F	N?	N	N	N	N	N	V	N	N	N	V?	N	N	
4F	N	N	N	N	N	N	V	N	N	N	N	–	N	–
5F	N	N	V	V	N	N	N	N	N	N	N	–	N	–
6F	N	N	N	V	N	N	N	N	N	N	N	–	–	–
7F	N	N	N	N	N	N	N	N	N	N	V	–	–	–
8F	N	N	N	N	N	N	V	N	N	N	N	–	–	–
9F	N	N	N	N	N	N	N	N	V	N	N	–	N	–
10F	N	N	N	N?	N	N	N?	N	N	N	N	N	N	N
11F	V	N	N	V	V	N	N	V	N	V	N	N	V	N
1D	V	V	N	V	N	N	V	N	N	N	N?	V	N	N?
2D	V	N	N	V!	N?	V	N	N	N?	V	N?	V?	N	N
5D	V	N?	V	N?	N	V	V	N	V	V	V	V?	N	
7D	N	N	V?	V	N	N	N	V	V	N?	V?	N	N	N
10D	N?	N	V	N?	V	N?	N	N	N	N?	N	V	N?	V
11D	N	V	N	V	V?	N	N	N	N	N	N	N	N	V?
12D	N	N?	N	V	N	V	N	N	N	N	N	N	N?	N
13D	N	N	N	N	N	N	N	N	N	V	N	N?	V	N
14D	N	N	N	V	V	N	N	N?	N	N	N	N	N	N
15D	N	N	V	V	N?	N	N	V?	V?	N?	N	N	N	N

ABBREVIATIONS: D, dermoid; F, fibroma; V, variable pattern; N, normal pattern.

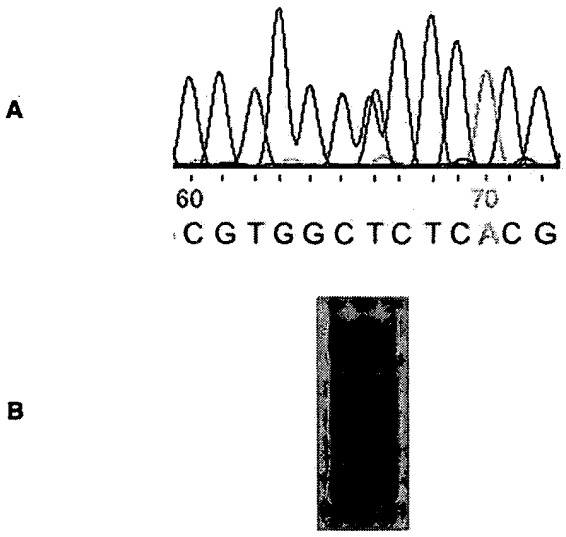

FIGURE 3. (A) Sequence analysis of an identified polymorphism on exon 12 of the PTCH gene in blood leukocytes of sample 3F. (B) SSCP pattern of the same sample.

particular genes are found. In fibromas, it appears that samples with LOH in the PTCH locus have low levels of PTCH expression, and only in two cases (6F, 9F) was PTCH expression greater than 70%. But the levels of SMO expression were frequently increased (samples 5F, 7F, 8F, 9F), suggesting that the pathway was activated, although GLI expression was increased only in one case (sample 4F). Similarly, in those dermoid samples in which PTCH was altered (as indicated by LOH in 9q22.3 locus) and the increased expression of SMO was observed, this was not accompanied by higher levels of GLI1 (sample 5D). However, in one dermoid case, both GLI1 and PTCH had increased expression.

DISCUSSION

Mutations in PTCH have been identified not only in Gorlin syndrome tumors but also in sporadic instances of the same tumors types, such as basal cell carcinoma (BCC), medulloblastoma, and meningioma, supporting the two-hit hypothesis. However, PTCH mutations have also been found in other malignancies, such as neuroectodermal tumors, breast carcinomas, squamous cell carcinoma, and trichoepithelioma.

Ovarian fibromas are only the third tumor by its incidence in Gorlin syndrome, but this neoplasm is so rare in the general population that the comparative increase indicates a strong association with the syndrome. Therefore, one aim of our study was to detect the involvement of PTCH and of the entire signaling pathway in the pathogenesis of this tumor. On the other hand, loss of heterozygosity for the PTCH region was also found in nonmalignant malformations (odontogenic cysts), so der-

TABLE 3. Expression levels of the analyzed pathway genes in ovarian fibromas and ovarian dermoids

	SMO	SHH	GLI1	PTCH
Fibroma				
1F	–	33%	–	–
2F	30%	14%	38%	–
3F	18%	–	–	35%
4F	–	44%	58%	–
5F	68%	35%	–	–
6F	19%	18%	–	59%
7F	94%	41%	–	–
8F	57%	64%	–	–
9F	61%	61%	–	71%
10F	24%	23%	–	–
11F	–	–	32%	–
Dermoids				
1D	12%	45%	55%	–
2D	12%	37%	–	62%
5D	–	34%	52%	–
7D	36%	67%	–	–
10D	45%	65%	–	–
11D	69%	29%	–	39%
12D	56%	74%	–	–
13D	–	18%	78%	78%
14D	28%	58%	21%	21%
15D	39%	57%	17%	17%

NOTE: – indicates very low expression or no expression. Gene expression calculated from equation based on ratio between max optical density of each sample, PO (housekeeping gene), and normal ovarian tissue (N) (OD max F,D/OD max PO(F,D)-OD max (N)/OD max PO(N).
ABBREVIATIONS: D, dermoid; F, fibroma.

moids were used in this study as representative of malformations in which mutations of PTCH and aberrations of Hedgehog/Patched signaling might occur.

Mutations and polymorphisms have been reported on all 23 exons of PTCH. No hot-spot regions can be identified for PTCH mutations, consistent with earlier observations that there is no correlation between genotype and phenotype expression. For polymorphic forms, however, literature data indicate clustering on a few exons in four regions of PTCH, and in our samples we also found a similar distribution. In addition, our study contributed a new polymorphism, 1647C→T, detected in ovarian fibroma.

The mechanism by which activation of the Hedgehog/Patched pathway leads to carcinogenesis is not entirely clear, but the pathway malfunctioning in some tumors has been demonstrated by mutations and/or aberrant expression of its genes. However, it is difficult to interpret the results of our study in terms of specific statements about the mechanisms of pathway action. It can be noted that high levels of SHH appear almost regularly, especially in dermoids, usually accompanied by increased expression of SMO. This might indicate that the tissue is constantly exposed to Shh signaling.

Also, alterations of PTCH indicated by LOH (including loss of the entire 9q22.3 region in two fibroma samples, 4F and 5F) barely affect its expression, compared with PTCH expression in a number of other samples. This would be consistent only with the interpretation that pathway aberrations mostly depend on Ptch protein functionality rather than abundance. And because it is also a target gene, its increased synthesis found in some samples can be attributed to pathway activation. However, it is interesting that GLI overexpression does not coincide with that of PTCH and is found only in a small proportion of cases. This leads to the conclusion that the proliferation of these tissues is not necessarily related to GLI activity.

LOH findings in the PTCH locus, and increased expression of several genes in the Hedgehog/Patched pathway, strongly suggest that the pathway is involved in both ovarian fibroma and dermoids. Although our observations do not clarify the mechanisms of pathway functioning, they may contribute to further investigations.

ACKNOWLEDGMENTS

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